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Characterization of *Salmonella* Toxin Released by Mitomycin C-Treated Cells

CLIFFORD W. HOUSTON,* FELIX C. W. KOO, AND JOHNNY W. PETERSON

Department of Microbiology, University of Texas Medical Branch, Galveston, Texas 77550

The enzyme-linked immunosorbent assay and the Chinese hamster ovary floating cell assay for cholera toxin have proven to be sensitive and reliable tests for determining the antigenic and biological characteristics of *Salmonella* toxin, respectively. The addition of mitomycin C to the culture media 3 h after inoculation resulted in increased amounts of *Salmonella* toxin in culture filtrates but had the reverse effect on cell sonic extracts. Our data suggested that the increased amounts of *Salmonella* toxin in culture filtrates caused by mitomycin C were due to cell lysis, resulting in the release of intracellular toxin, rather than to an increase in the synthesis of *Salmonella* toxin. The biological activity of *Salmonella* toxin was heat labile at 100°C. The antigenic structure of the toxin appeared to remain intact after exposure to temperatures as high as 100°C but was altered somewhat when the toxin was subjected to autoclaving. The toxin had an isoelectric point in the pH range from 4.3 to 4.8 and an estimated molecular weight which appeared to be more than 110,000. With the exception of the range for its isoelectric point, its molecular weight, and its low concentration in filtrates and sonic extracts, *Salmonella* toxin was very similar in biological and antigenic characteristics to cholera toxin. The antigenic and biological assays described here provide an effective basis for extending our study of *Salmonella* toxin.

Several genera of enteric pathogenic bacteria associated with diarrheal disease release enterotoxins that are responsible for the loss of fluid and electrolytes from the intestinal mucosa. The best characterized of these enterotoxins are the heat-labile toxins of *Vibrio cholerae* and *Escherichia coli* (1, 3-5, 11, 12). The mechanism of action of these antigenically related toxins involves stimulation of adenylate cyclase and elevation of intracellular levels of cyclic adenosine monophosphate. Guerrant and co-workers (7) showed that *E. coli* and *V. cholerae* enterotoxins could elevate intracellular concentrations of cyclic adenosine monophosphate in Chinese hamster ovary (CHO) cells; these changes in cyclic nucleotide metabolism were correlated with morphological alterations in the cells, thus forming the basis for a simple assay to detect these enterotoxins.

Extensive investigations of *V. cholerae* and *E. coli* enterotoxins led to the concept that other enteric bacteria may also elaborate adenylate cyclase-stimulating toxins. In 1975 Koupal and Deibel (10) described an enterotoxic factor from *Salmonella* species that caused fluid accumulation in suckling mice. Independently, Sandefur and Peterson (17) described a skin permeability factor, which is now referred to as *Salmonella* toxin; this toxin was heat labile and altered vascular permeability in the skin of adult rabbits in a manner indistinguishable from the alteration caused by *V. cholerae* and *E. coli* enterotoxins. Recently, this toxin was shown to possess enterotoxic activity in adult rabbits (Peterson et al., submitted for publication).

Sandefur and Peterson (18) reported the neutralization of the vascular permeability activity of *Salmonella* toxin with monospecific cholera antitoxin. In addition, *Salmonella* toxin was shown to be responsible for the elongation of CHO cells in a manner identical to the elongation caused by cholera toxin.

The purpose of this investigation was to study the effect of mitomycin C (MTC) on synthesis and release of *Salmonella* toxin, as well as its biochemical characteristics, by using two very sensitive and reliable assays for monitoring the biological activity and antigenic presence of this toxin. The biological activity of *Salmonella* toxin was measured with a CHO floating cell assay, which was a modification of the CHO floating cell assay for cholera toxin devised by Nozawa and co-workers (14). The antigenic presence of the toxin was monitored by an enzyme-linked immunosorbent assay (ELISA), which was a slightly modified version of the method described by Voller et al. (19).

MATERIALS AND METHODS

Organisms. *Salmonella typhimurium* strains SL1027 and SR11 were supplied by Samuel Formal, Walter Reed Army Medical Center, and L. Joe Berry, University of Texas at Austin, respectively. Four clinical isolates of *Salmonella enteritidis* (strain 9630 serotype Newport, strain 9186 serotype Newport, strain 10016 serotype Javiana, and strain 8994 serotype Braenderup) were supplied by the Houston Health Department.

Preparation of cultures. Flasks containing 50 ml of Casamino Acids-yeast extract (CYE) medium (13) were inoculated in duplicate with one of the following strains of *Salmonella*: *S. enteritidis* 9186, 10016, or 9884 or *S. typhimurium* SL1027 or SR11 from a slant of CYE agar. *S. enteritidis* strain 9630 was inoculated into a 250-ml flask containing 100 ml of Trypticase soy broth. These flasks were incubated with mild shaking (100 rpm) at 37°C for 3 h before MTC was added to a final concentration of 0.5 μg/ml (13) to one of the duplicate flasks. The flasks were then incubated for an additional 21 h with mild shaking at 37°C.

Preparation of filtrates. After the 24-h incubation period, the cultures were centrifuged at 12,000 × *g* for 10 min, and the supernatant fluids were filtered through 0.20-μm sterile filter units (Nalgene Labware Div., Nalge/Sybron Corp.). The filtrates were placed into sterile plastic tubes for storage at 4°C. One filtrate of *S. enteritidis* 9630, which was grown in the presence of MTC (designated strain 9630-MTC), was concentrated 15-fold by dialysis in 20 M Carbowax (Union Carbide); this was followed by dialysis of the concentrate in phosphate diluent (PD) buffer (pH 7.0), which contained (per liter) 8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, and 0.2 g of KH_2PO_4.

Sonication. The *Salmonella* cells were removed from the filtrates, washed one time with 40 ml of PD buffer, and resuspended in 10 ml of PD buffer before sonication. Each preparation was submerged in an ice bath and sonicated with a Branson sonifier equipped with a microtip at 70 W for 5 min. The sonic extracts were centrifuged at 15,000 × *g* for 15 min and sterilized by filtration through 0.2-μm Nalgene filter units. The resulting supernatant fluids were assayed for antigenic and biological activities by using the ELISA and the CHO floating cell assay, respectively.

Preparation of sonicated cultures. Flasks (500 ml) containing 100 ml of CYE medium were inoculated in duplicate with 100 μl of one of the following strains of *Salmonella*: *S. typhimurium* SR11 or SL1027 or *S. enteritidis* 8994 from an overnight CYE broth culture. The flasks containing the inoculated medium were incubated with mild shaking (100 rpm) at 37°C for 5 h before MTC (final concentration, 0.5 μg/ml) was added to one of the duplicate flasks. The flasks were incubated for an additional 21 h with mild shaking at 37°C. After the incubation period, the flasks were placed on ice immediately. Both the control culture and the culture grown with MTC were divided into two 50-ml portions. A 50-ml sample of the control culture and a 50-ml sample of the MTC-treated culture of each strain were sonicated before centrifugation and processed as described above, with the exception that the microtip was not used. The remaining 50-ml portions of the control culture and the MTC-treated culture of each strain were centrifuged. Filtrates and sonic extracts were prepared as described above. A protein determination (2) was conducted on filtrates, sonic extracts, and whole sonicated cultures.

ELISA. On the basis of the observation that *Salmonella* toxin shares antigenic determinants with cholera toxin, we used the ELISA to detect *Salmonella* toxin, using a specifically purified antibody to cholera toxin. We used the method of Voller et al. (19), with slight modifications, to detect *Salmonella* toxin antigen. Crude filtrates containing *Salmonella* toxin were mixed with equal volumes of carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6), and 200-μl portions of each mixture were allowed to adsorb to the wells of a polystyrene microtiter plate (Dynatech Laboratories, Inc.) during an overnight incubation in a humid chamber at room temperature. The washing procedure used involved removing the contents of the wells, filling the wells with phosphate-buffered saline–Tween buffer (pH 7.4), and letting the wells stand for 3 min. This washing procedure was repeated three times. A 200-μl volume of a cholera antitoxin (stock solution, 1.5 mg/ml), which was produced in rabbits and specifically purified with a cholera toxin immunoadsorbent (8, 15), was added in a 1:50 dilution with phosphate-buffered saline–0.05% Tween 20 buffer to each well and allowed to react with the adsorbed toxin for 2 h. The wells were then washed as described above. A 200-μl volume of a 1:1,000 dilution of goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Miles Laboratories, Inc.) in phosphate-buffered saline–Tween buffer was added to each well and allowed to react with the cholera antitoxin for 2 h. Finally, after the wells were washed, 200 μl of the colorless substrate *p*-nitrophenyl phosphate (1 mg/ml in carbonate buffer, pH 9.6) was added to each well, and the reaction was allowed to progress at 25°C for 30 min. The reaction was stopped by adding 50 μl of 3 M NaOH. The amount of *p*-nitrophenol released was measured spectrophotometrically at 400 nm. Dilutions of purified cholera toxin in carbonate buffer were utilized as standards in the ELISA, and the antigen contents of crude *Salmonella* filtrates, sonic extracts, and fractions were determined and plotted against the cholera toxin standards. The *Salmonella* toxin values were expressed as cholera toxin equivalent units and reflected total antigen contents.

CHO floating cell assay. We used a modification of the CHO floating cell assay devised by Nozawa and co-workers (14). Each well of a plastic multidish (Linbro Plastics) was inoculated with 2.0×10^5 CHO cells per cm^2. The cells formed a monolayer after 24 h of incubation at 37°C in an atmosphere containing 5% CO_2. After each monolayer was rinsed with PD buffer, fresh medium with or without toxin was added, and the cells were incubated for an additional 18 h. During this time, the proliferating CHO cells that were not subjected to the toxin floated off the monolayers into the medium, whereas the wells that were inoculated with the toxin had more cells attached to their monolayers and fewer floating cells. The medium (0.5 ml) containing the floating cells was removed, and each monolayer was washed once with 1 ml of PD buffer. The medium and the wash were added to accuvettes containing 8.5 ml of PD buffer before the floating cells were counted with a Coulter Counter (Fig. 1).

Heat study. A crude filtrate from an *S. enteritidis* strain 9630 culture which was treated with MTC was tested for the heat stability of both its antigenic activities and its biological activities. Sealed tubes containing 1-ml portions of filtrate were incubated for 1 h at

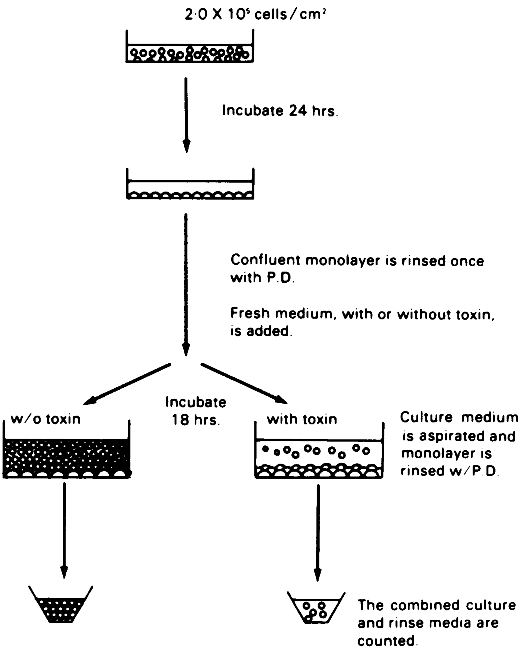

FIG. 1. *Diagramatic scheme of the modification of the CHO floating cell assay. P.D., PD buffer; w/o, without; w/, with.*

one of the following temperatures: 4, 37, 50, or 65°C. A sealed tube containing a 1-ml portion of *Salmonella* filtrate was also incubated at 100°C for 10 min, and another 1-ml portion of *Salmonella* filtrate was subjected to autoclaving at 121°C for 15 min. In addition, sonic extracts and filtrates of clinical isolates of *S. enteritidis* 8994, 10016, and 9186 and *S. typhimurium* SR11 and SL1027 were heated by autoclaving for 15 min. The autoclaved filtrates and sonic extracts were tested for residual biological activity by the CHO floating cell assay and for antigenic stability by the ELISA.

Column chromatography. Partial purification of the toxin from a crude filtrate of MTC-treated *S. enteritidis* 9630 was accomplished by gel filtration through a calibrated column (1.5 by 100 cm) of Sephadex G-150 (Pharmacia Fine Chemicals, Inc.) equilibrated with PD buffer (pH 7.0). Fractions (2 ml) were collected, and optical density was monitored at 280 nm. The column was calibrated with the following protein standards: 5 mg of ribonuclease A (13,700 daltons), 5 mg of chymotrypsinogen (25,000 daltons), 5 mg of ovalbumin (45,000 daltons), 5 mg of bovine serum albumin (67,000 daltons), 5 mg of aldolase (158,000 daltons), 5 mg of ferritin (440,000 daltons), and 1 mg of blue dextran (2,000,000 daltons). To estimate the molecular weight of the *Salmonella* toxin, the elution volumes of the toxin fractions containing maximum biological activity, as determined by the CHO floating cell assay, were compared with the optical density peaks of the protein standards with known molecular weights.

Preparative isoelectric focusing. Approximately 1 mg of dialyzed filtrate from *S. enteritidis* 9630-MTC was applied to a preparative isoelectric focusing flatbed containing Sephadex G-75 superfine. The filtrate was subjected to 16 h of isoelectric focusing at 8 W (constant power) and 4°C (ampholine range, pH 3.5 to 5.0). The flat-bed gel was divided into 30 fractions, the pH in these fractions was measured with a surface electrode, and these fractions were dialyzed against PD buffer to remove the ampholines. The antigenic activities and the biological activities of the resulting fractions were monitored by the ELISA and the CHO floating cell assay, respectively.

RESULTS

A standard curve was devised by using different concentrations of purified cholera toxin in the CHO floating cell assay (Fig. 2). This assay was capable of detecting as little as 0.01 ng of cholera toxin. As the concentration of cholera toxin was increased, a smaller number of CHO floating cells was present in the culture medium; however, a larger number of floating cells was obtained when a monolayer was not exposed to cholera toxin. Photomicrographs of fixed and stained cells on monolayers after removal of the floating cells are shown in Fig. 3. Figure 3A shows normal CHO cells, whereas Fig. 3B shows cells that were treated with 0.11 ng of cholera toxin. The toxin-treated cells appeared elongated compared with the normal CHO cells.

A standard curve determined by using different concentrations of purified cholera toxin was utilized in the ELISA (Fig. 4). As Fig. 4 shows, the ELISA was capable of detecting quantities as small as 0.37 ng of cholera toxin. The amount of *p*-nitrophenol released was directly proportional to the amount of antigen present over a 2-log range. The same standard curve was obtained when two different concentrations of alkaline phosphatase-labeled goat anti-rabbit immunoglobulin were used in the ELISA. We per-

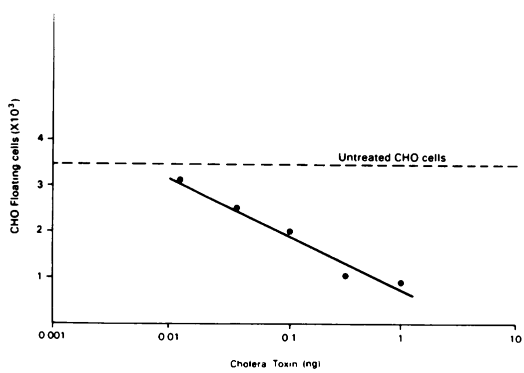

FIG. 2. *Standard curve determined by using different concentrations of purified cholera toxin in the CHO floating cell assay. The solid line shows the decrease in the number of floating cells as the concentration of cholera toxin was increased.*

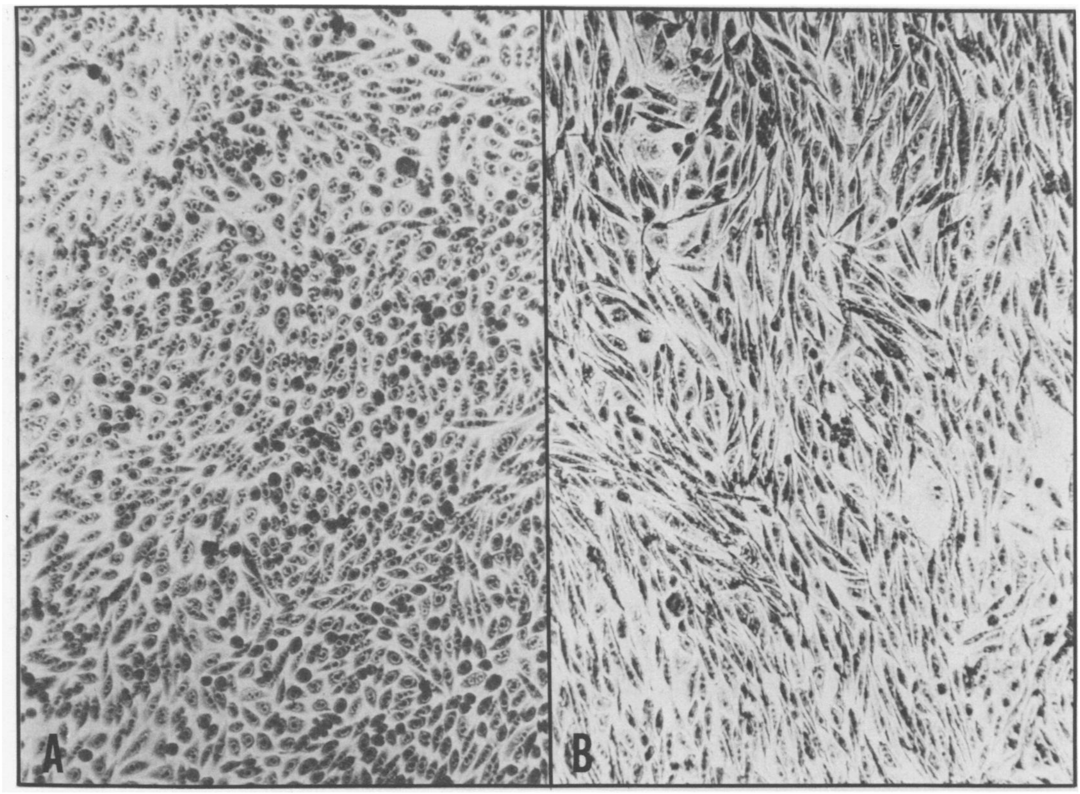

FIG. 3. *Photomicrographs of fixed and stained normal cells (A) and cholera toxin-treated cells (B) of monolayers after the removal of floating cells. ×300.*

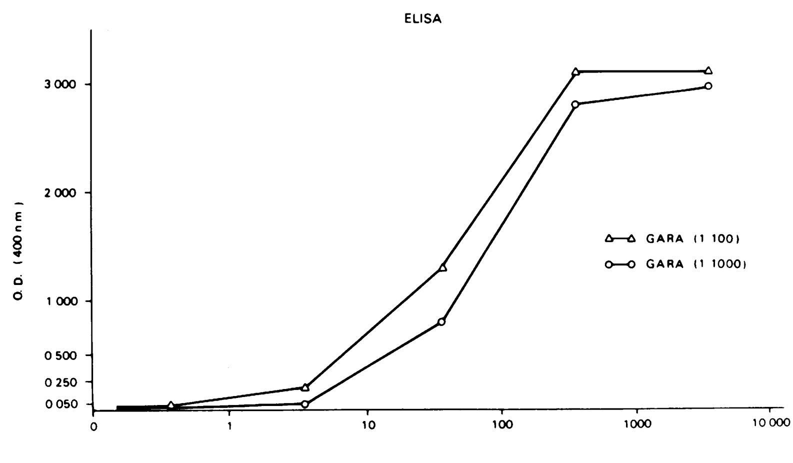

FIG. 4. *Standard curve determined by using different concentrations of purified cholera toxin in the ELISA. GARA, Alkaline phosphatase-labeled goat anti-rabbit antibody conjugate; O.D. (400 n.m.), optical density at 400 nm.*

formed an experiment to determine the specificity of the ELISA (Table 1). Various antigens were assayed with and without specifically purified cholera antitoxin in the second step of the ELISA. When cholera antitoxin was used in the ELISA, we obtained readings higher than the readings obtained when no cholera antitoxin was present.

Molina and Peterson (13) demonstrated that the addition of 0.5 μg of MTC per ml of the

TABLE 1. *Specificity of the ELISA*

Antigen	Optical density at 400 nm: With cholera antitoxin[a]	Optical density at 400 nm: Without cholera antitoxin[b]
Choleragen (20 μg)	3.201	0.070
S. typhimurium SL1027 filtrate (MTC)	0.285	0.000
S. typhimurium SR11 filtrate (MTC)	0.437	0.004
S. enteritidis 8994 filtrate (MTC)	0.337	0.006
S. typhimurium SL1027 sonic extract	0.155	0.000
S. typhimurium SR11 sonic extract	0.322	0.018
S. enteritidis 8994 sonic extract	0.226	0.011
CYE medium control	0.012	0.001
CYE-MTC medium control	0.043	0.000

[a] Cholera antitoxin was used in the second step of the ELISA.

[b] Phosphate-buffered saline-Tween was used in the second step of the ELISA.

culture media after a 3-h incubation period caused the *Salmonella* strains to release an increased amount of *Salmonella* toxin into filtrates, as demonstrated by the elongation of CHO cells. We conducted a similar experiment by using the CHO floating cell assay and the ELISA to detect *Salmonella* toxin biological activity and antigen activity, respectively, in cell sonic extracts and culture filtrates (Table 2). The *Salmonella* toxin concentration was expressed as cholera toxin equivalent units (in nanograms per milliliter) since the CHO floating cell assay and the ELISA values obtained for the *Salmonella* preparation were plotted against cholera toxin standard curves. The addition of MTC increased the amount of *Salmonella* toxin in the filtrates, as determined by both the CHO floating cell assay and the ELISA. A protein determination revealed increased amounts of protein in the filtrates after MTC was added. When the sonic extracts were assayed for the presence of *Salmonella* toxin by the CHO floating cell assay and the ELISA, we found higher levels in the control cultures than in the cultures

TABLE 2. *Effect of MTC on the presence of Salmonella toxin in filtrates and sonic extracts of Salmonella strains*

Strain	Prepn	Protein concn (mg/ml)	CHO floating cell assay: ng/ml	CHO floating cell assay: ng/mg of protein	ELISA: ng/ml	ELISA: ng/mg of protein
SR11	Filtrate[a]	0.003	0.01	3.33	2.50	833.33
	Sonic extract[b]	0.794	1.84	9.27	5.40	6.80
	Whole sonicated culture[c]	0.989	0.64	0.58	2.97	3.00
SR11 (MTC treated)	Filtrate	0.275	7.36	6.69	3.10	11.27
	Sonic extract	0.188	0.01	0.05	2.75	14.63
	Whole sonicated culture	0.475	9.16	3.96	3.65	7.68
8994	Filtrate	0.005	0.58	128.00	2.40	480.00
	Sonic extract	0.705	1.88	12.99	7.70	10.92
	Whole sonicated culture	0.836	3.86	4.61	3.12	3.73
8994 (MTC treated)	Filtrate	0.170	5.24	30.82	3.34	19.65
	Sonic extract	0.172	1.26	7.33	3.30	19.18
	Whole sonicated culture	0.108	0.72	6.66	2.60	24.07
SL1027	Filtrate	0.007	0.01	1.43	1.50	214.29
	Sonic extract	0.584	12.48	21.37	5.80	9.93
	Whole sonicated culture	0.711	6.40	9.00	3.35	4.71
SL1027 (MTC treated)	Filtrate	0.118	8.38	71.02	3.05	25.85
	Sonic extract	0.153	3.48	22.75	2.50	16.34
	Whole sonicated culture	0.132	5.60	42.42	2.92	22.12

[a] The filtrate values represent cholera toxin equivalent units (as determined by the CHO floating cell assay and the ELISA) for membrane-filtered (Millipore Corp.) supernatants of centrifuged 50-ml CYE broth cultures of *Salmonella* strains. The data are expressed as cholera toxin equivalent units per milliliter.

[b] The sonic extract values represent cholera toxin equivalent units (as determined by the CHO floating cell assay and the ELISA) for the cell pellets of centrifuged CYE broth cultures of *Salmonella* strains which were suspended in 50 ml of PD buffer, sonicated, and membrane filtered (Millipore Corp.). The data are expressed as cholera toxin equivalent units per milliliter.

[c] Whole sonicated culture values represent cholera toxin equivalent units (as determined by the CHO floating cell assay and the ELISA) for 50-ml portions of CYE broth cultures of *Salmonella* strains which were first sonicated and then centrifuged. The data are expressed as cholera toxin equivalent units per milliliter.

exposed to MTC. Protein levels for the sonic extracts were also higher in the control cultures than in the cultures grown in the presence of MTC. This suggested that *Salmonella* toxin and a large amount of nontoxin protein were released from the cells into the medium when MTC was added. We attempted to estimate the total amounts of *Salmonella* toxin and protein in whole sonicated CYE broth cultures of *Salmonella* strains. Whole sonicated cultures exposed to MTC contained smaller amounts of protein than control whole sonicated cultures. When *Salmonella* toxin values for the whole sonicated cultures (as determined by the CHO floating cell assay and the ELISA) were divided by the respective protein values (specific biological activity), the amount of *Salmonella* toxin per milligram of protein appeared to be increased in the presence of MTC.

To determine whether MTC caused an increase in synthesis of *Salmonella* toxin, we performed a further analysis of the data in Table 2 (Table 3). The amounts of protein lost from the sonic extracts were greater than the amounts which appeared in the filtrates. In addition, the amount of *Salmonella* toxin biological activity gained in the filtrate, as determined by the CHO floating cell assay, was almost equal to the amount lost in the sonic extract of *S. typhimurium* SL1027. However, *Salmonella* toxin antigen was lost from sonicated *Salmonella* cells in amounts greater than the amounts of gained in the filtrates. The concentrations of *Salmonella* toxin and protein were expressed as total microgram amounts to illustrate the relatively small amounts of toxin present in the filtrates and sonic extracts compared with the overall protein levels.

TABLE 3. *Net increases and decreases in Salmonella toxin and protein levels in filtrates and sonic extracts of three Salmonella strains grown in the presence of MTC, compared with control cultures*[a]

Strain	Prepn	Amt of protein (μg)[b]	CHO floating cell assay (μg)[c]	ELISA (μg)[c]
SR11	Filtrate	+13,600	+0.3675	+0.0300
	Sonic extract	−30,300	−0.0915	−0.1325
8994	Filtrate	+8,250	+0.2330	+0.0470
	Sonic extract	−26,650	−0.0310	−0.2200
SL1027	Filtrate	+5,550	+0.4160	+0.0775
	Sonic extract	−29,550	−0.4500	−0.1650

[a] A positive value indicates a net increase in the level of *Salmonella* toxin or protein. Positive values for the filtrates were obtained by subtracting the protein or cholera toxin equivalent value (in micrograms per milliliter) for the control filtrate of each strain from the protein or cholera toxin equivalent value (in micrograms per milliliter) for the MTC-treated filtrate of the same strain. This value was then multiplied by the total volume to obtain the positive values listed for the filtrates. A negative value indicates a net decrease in the level of *Salmonella* toxin or protein. Negative values for the sonic extracts were obtained by the method described above.

[b] Expressed as the amount of total protein in 50 ml.

[c] Expressed as total cholera toxin equivalent units (in micrograms) in 50 ml.

The addition of MTC to CYE broth medium containing exponentially growing cultures of *Salmonella* strains appeared to cause cell lysis and the release of *Salmonella* toxin, as well as the release of other intracellular components. A decrease in turbidity of 50% or more, as determined by Klett readings, and a drop in viability of at least 98.6% indicated that cell lysis may have occurred. Finally, the nucleic acid contents of *Salmonella* strains were reduced by at least 72% (as determined by 260- and 280-nm ultraviolet light readings, which were plotted from a nomograph) in sonic extracts of cells which had been exposed to MTC, compared with sonic extracts of the control cultures. Sonication reduced the number of viable *Salmonella* cells by at least 95% in CYE broth cultures of the five *Salmonella* strains tested.

A reduction in the biological effect on CHO cells of approximately 93% was observed when MTC filtrates of *S. enteritidis* 9630 were heated to 100°C (Fig. 5). The antigenic structure of the *Salmonella* toxin, as recognized by the ELISA, remained intact when the toxin was subjected to heating at this same temperature.

Filtrates and sonic extracts of five other strains of *Salmonella* (*S. enteritidis* 8994, 10016, and 9186 and *S. typhimurium* SR11 and SL1027) were tested for *Salmonella* toxin heat lability. We found that these preparations lost most of their biological activity (95%) when they were subjected to autoclaving for 15 min, as determined by the CHO floating cell assay. Autoclaving resulted in average decreases in ELISA ab-

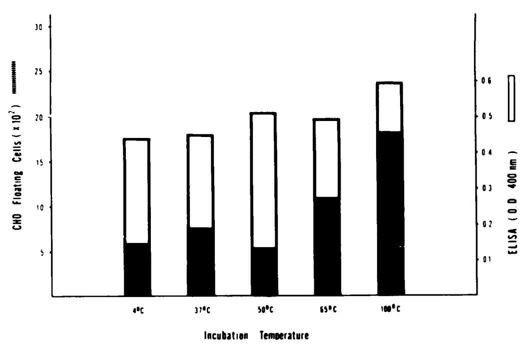

FIG. 5. *Effect of heat on the biological activity and antigenicity of Salmonella toxin. A separate sample of culture filtrate containing Salmonella toxin was incubated at each of the temperatures indicated for 1 h, with the exception that incubation at 100°C was for 10 min. The open bars represent ELISA values, and the shaded bars represent numbers of CHO floating cells. O.D., Optical density.*

sorbance values of 35% for the *Salmonella* toxin from the filtrates and 64% for the *Salmonella* toxin from the sonic extracts (Table 4).

The application of approximately 1 mg of filtrate from *S. enteritidis* 9630-MTC to a preparative isoelectric focusing flat-bed gel with an ampholine range of pH 3.5 to 5.0 resulted in the data shown in Fig. 6. The *Salmonella* toxin antigen as determined by the ELISA was located in the range from pH 4.3 to pH 4.8. The biological activity of the *Salmonella* toxin antigen as measured by the CHO floating cell assay was located in the same region, despite the absence of a significant protein peak.

Partial purification of the *Salmonella* toxin from the culture medium and other *Salmonella* products was achieved by applying 3 ml of a 15-fold concentrated, cell-free filtrate from *S. enteritidis* 9630 to a Sephadex G-150 column. Figure 7 shows the elution pattern of this concentrated filtrate. When fractions were tested by the ELISA and the CHO floating cell assay for antigenic activity and biological activity, respectively, we found both antigenic activity and biological activity associated with the first peak.

DISCUSSION

The addition of MTC increased the amount of *Salmonella* toxin present in filtrates, as determined by the ELISA and the CHO floating cell assay. In addition, protein contents increased in *Salmonella* filtrates due to the addition of MTC. There are at least two possible explanations for this MTC phenomenon. Isaacson and Moon (9) postulated that MTC-induced heat-labile toxin synthesis in enterotoxigenic strains of *E. coli* was due to plasmid gene derepression. On the other hand, Gemski et al. (6) have reported the cellular release of heat-labile enterotoxin from *E. coli* by bacteriophage induction. The data from our studies tend to support the latter mechanism. A sharp decrease in the turbidities of *Salmonella* broth cultures grown in the presence of MTC was detected by Klett readings. This was confirmed by a drastic decrease in the number of viable cells present in the cultures to which MTC had been added. The nucleic acid contents of *Salmonella* cells exposed to MTC was reduced by 72% compared with the control *Salmonella* cultures. These data indicated that nucleic acid (an internal marker) was released as a result of cell lysis after MTC was added. We believe that MTC increases the concentration of extracellular *Salmonella* toxin by lytic induction. Studies are presently being conducted to determine whether cell lysis is due to MTC induction of bacteriophage (Peterson, Houston, and Koo, Infect. Immun., in press).

We can speculate that MTC was responsible for the following two independent phenomena: the selective inhibition of nontoxin protein synthesis and the lytic release of *Salmonella* toxin. More *Salmonella* toxin biological activity was detected in the control culture sonic extracts than in the control culture filtrates, as determined by the CHO floating cell assay. In contrast, *Salmonella* strains grown in the presence of MTC had more *Salmonella* toxin biological activity in their filtrates than in their cell sonic extracts. When the biological activities of whole sonicated cultures were determined, more toxin was detected in the control cultures of two of the three strains tested (Table 2). The specific biological activity of *Salmonella* toxin (cholera toxin equivalent units per milligram of protein) appeared to increase in the presence of MTC, as indicated by the data for the whole sonicated cultures. This apparent increase in specific biological activity was due to a reduction in the amount of protein found in whole sonicated cultures which were exposed to MTC rather than to an enhancement of *Salmonella* toxin synthesis. The reduction in total protein content in MTC-treated whole sonicated cultures greatly exceeded the small reductions detected for *Salmonella* toxin. Data from a previous study (Peterson et al., in press) indicated that *Salmonella* toxin was synthesized early during the growth

TABLE 4. *Percent decrease in cholera toxin equivalents of Salmonella toxin in filtrates and sonic extracts of five Salmonella strains after autoclaving for 15 min*[a]

Strain	Prepn	% Decrease in: ELISA[b]	% Decrease in: CHO floating cell assay[c]
9186	Filtrate	53	85
	Sonic extract	74	
8994	Filtrate	39	73
	Sonic extract	42	98.5
10016	Filtrate	28	96
	Sonic extract	33	99.5
SR11	Filtrate	30	96
	Sonic extract	89	99.9
SL1027	Filtrate	26	98.2
	Sonic extract	81	99.5

[a] Percent decrease was calculated by dividing the cholera toxin equivalents of MTC-treated filtrates subjected to autoclaving for 15 min by the cholera toxin equivalents of MTC-treated filtrates which were not subjected to autoclaving and multiplying by 100. This value was subtracted from 100%, and the resulting percentage was assumed to be the percent decrease.

[b] As determined by the ELISA, the average percent decrease for filtrates was 35% and the average percent decrease for sonic extracts was 64%.

[c] As determined by the CHO floating cell assay, the average percent decrease for filtrates was 90% and the average percent decrease for sonic extracts was 99%.

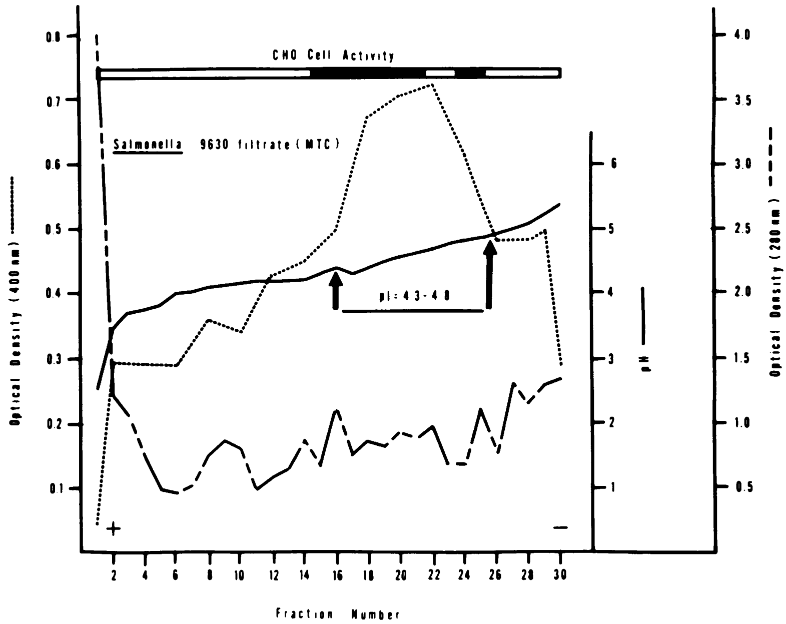

FIG. 6. *Isoelectric focusing of 1 mg of culture filtrate containing Salmonella toxin in a preparative flat-bed gel with an ampholine range from pH 3.5 to 5. The pH and absorbancy at 280 nm were plotted against fraction number. ELISA values (dotted line) were measured at an optical density of 400 nm. pH values are represented by the solid line, the protein values are represented by the dashed line, and the CHO cell activity is represented by the solid bar (equivalent to an average of 2.36 ng of cholera toxin equivalent units per ml).*

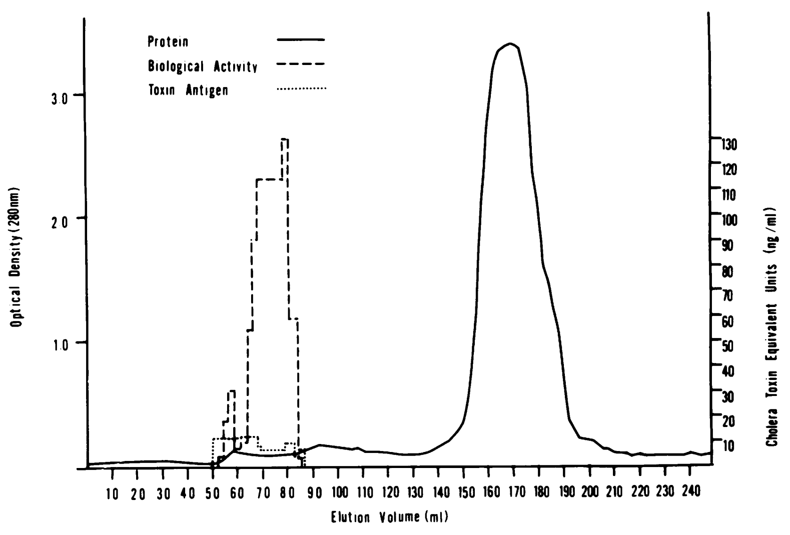

FIG. 7. *Chromatography of a 15-fold-concentrated crude filtrate of S. enteritidis 9630-MTC on Sephadex G-150. The optical density at 280 nm is represented by the solid line. The column was equilibrated and eluted with PD buffer (pH 7.0). The biological activity (dashed line) and the toxin antigen (dotted line) were monitored with the CHO floating cell assay and the ELISA, respectively. Values for both assays are expressed as cholera toxin equivalent units (in nanograms per milliliter).*

cycle of *S. typhimurium* SL1027, reaching maximum levels in control cell sonic extracts at 12 h after the initial inoculation, whereas nontoxin protein levels (unpublished data) of control cell sonic extracts continued to increase after 36 h. When MTC was added at 8.5 h after the initial inoculation, cell lysis occurred at 14 h, and this inhibited protein synthesis. The reduction in nontoxin protein levels compared with toxin levels in MTC-treated sonic extracts also resulted in an increase in specific activity of the toxin. These data were interpreted as indicating selective inhibition of protein synthesis.

Similarly, more *Salmonella* toxin antigen was detected by the ELISA in the control culture sonic extracts than in the control culture filtrates (Table 2). When *Salmonella* strains were grown in the presence of MTC, more *Salmonella* toxin antigen was present in the filtrates than in the cell sonic extracts. It is interesting that for two of the three *Salmonella* strains tested, whole sonicated control cultures contained slightly more *Salmonella* toxin antigen than whole sonicated cultures exposed to MTC. If MTC were responsible for an increase in toxin synthesis, one would expect to find a significantly greater amount of *Salmonella* toxin in MTC-treated whole sonicated cultures. In view of the fact that nucleic acid release was due to MTC-induced lysis of *Salmonella* cells, *Salmonella* toxin antigen may be released by the same mechanism. When expressed as toxin antigen per milligram of protein, *Salmonella* toxin antigen from whole sonicated cultures appeared to have more *Salmonella* toxin in MTC-treated whole sonicated cultures than in control whole sonicated cultures. As described above for specific biological activity, the increase in toxin antigen per milligram of protein may be due to a reduction in the amount of protein found in whole sonicated cultures treated with MTC rather than to an enhancement of toxin synthesis.

Sonication of *Salmonella* cells, which produced the toxin found in the filtrates, released more protein in the control cultures than in the cultures grown in the presence of MTC (Table 2). Sonication of whole broth cultures confirmed that control culture cells contained more protein than cells grown in the presence of MTC. The data in Table 2 suggested that some of the protein was released into the medium as a result of cell lysis by the addition of MTC, as indicated by the amounts of protein detected in the filtrates of *Salmonella* strains grown in the presence of the drug. In most cases, the *Salmonella* toxin levels for the whole sonicated cultures were less than the levels for the combined filtrates and sonic extracts of each strain (Table 2). Perhaps the whole sonicated cultures in broth were more sensitive to proteinase activity than the sonic extracts in PD buffer. Another possible explanation may be that sonication of broth cultures releases toxin that becomes bound to nontoxin molecules, thereby altering the biological and antigenic characteristics of the molecules. Although we are presently unable to define the exact cause of this phenomenon, we are positive that the toxin was altered in some manner since the protein values for the control filtrates and sonic extracts were approximately equal to the protein values for the whole sonicated cultures.

We determined that more protein was lost from the cell sonic extracts than was gained in the filtrates in response to MTC (Table 3). This was an indication of protein release rather than de novo protein synthesis. In Table 3, the data for *S. typhimurium* SL1027 demonstrate that the biological activity gained in the filtrates was almost equal to the amount lost from the sonic extracts, which suggested MTC-mediated toxin release rather than toxin synthesis. Perhaps the sonic extracts of the two other *Salmonella* strains described in Table 3 were partially denatured by heat generated during sonication. This could account for the lower net decrease in biological activity found in the sonic extracts compared with the net increase of activity in the filtrates.

Table 3 indicates that the net decrease in *Salmonella* toxin antigen levels in sonic extracts was larger than the net increase in *Salmonella* toxin antigen detected in the filtrates of the same MTC-treated strains. This was interpreted as being due to toxin release rather than toxin synthesis. Perhaps some of the *Salmonella* toxin released by MTC-mediated cell lysis was adsorbed to the resulting particulate debris in the medium, which may have accounted for the lower increase in *Salmonella* toxin levels in the filtrates. Based on the data presented above, MTC may have been responsible for a type of selective inhibition of protein synthesis in which the rate of toxin synthesis was not altered but other nontoxin protein synthesis was affected. In addition, we present evidence which favors the hypothesis that *Salmonella* toxin was released in significant amounts when the cells were lysed in the presence of MTC or as a result of sonication.

The heat stability study indicated that heating the toxin-containing filtrate from *S. enteritidis* 9630-MTC to 100°C for 10 min caused a 93% reduction in the biological effect of the filtrate on CHO cells, but that the antigenic structure of the toxin (as recognized by the ELISA) remained intact. It was only under extreme conditions, such as autoclaving, that the antigenic structure of the toxin molecule was altered. In addition, filtrates and sonic extracts of five other *Salmonella* strains (*S. typhimurium* SL1027 and SR11 and *S. enteritidis* 9186, 10016, and 8994) were each heated by autoclaving for 15 min. The biological activities of the filtrates and sonic extracts from these five strains were drastically reduced (by 95%) after autoclaving, as determined by the CHO floating cell assay. This indicated that the intracellular *Salmonella* toxin found in the sonic extracts was heat labile, as was the toxin found in the filtrates. The absorbance in the ELISA for *Salmonella* toxin in the filtrates of the five *Salmonella* strains mentioned above was decreased an average of 35% by autoclaving, whereas sonic extracts of the same strains experienced an average decrease of 64% as ascertained by the ELISA. Perhaps the antigenic structure of the *Salmonella* toxin in the filtrates was less heat sensitive than the structure of the toxin in the sonic extracts because of an association with nontoxin molecules in the filtrates. This may have had some protective function when the toxin was subjected to heating. Further studies of this phenomenon must be conducted before any definitive explanation can be made. The heat lability of *Salmonella* toxin found in this study confirmed the previous data obtained by Sandefur and Peterson (17).

Partial purification of the *Salmonella* toxin from a crude filtrate of *S. enteritidis* 9630 grown with MTC was accomplished by gel filtration through a calibrated column of Sephadex G-150 equilibrated with PD buffer (pH 7.0). A molecular weight of at least 110,000 for *Salmonella* toxin was calculated by using known protein standards. This was within acceptable range of the molecular weight calculated by Sandefur and Peterson for *Salmonella* toxin (90,000) (17). This toxin was associated with the first small peak of the elution pattern, which was near the void volume of the Sephadex G-150 column, as determined by the CHO floating cell assay and the ELISA. A more definitive estimate of the molecular weight of *Salmonella* toxin will be made in the future by using other methods.

Preparative flat-bed isoelectric focusing has proven to be extremely useful in terms of isolating the *Salmonella* toxin and characterizing its isoelectric point. The *Salmonella* toxin was negatively charged and had an isoelectric point in the pH range of 4.3 to 4.8, as determined by the ELISA and the CHO floating cell assay. These data coincide precisely with the isoelectric point of *Salmonella* delayed permeability factor reported by Peterson and Sandefur (16). Preparative isoelectric focusing has the possibility of being used as a final purification step for future work because of its ability to separate *Salmonella* toxin from other molecules, based on the isoelectric point of the toxin. Unfortunately, the protein concentrations of fractions containing *Salmonella* toxin were too small to be detected as bands by Coomassie blue G250 staining of polyacrylamide gels. The *Salmonella* toxin was present in nanogram quantities in filtrates, which is below the detectable range for stains such as Coomassie blue. This explains why the antigenic and biological activities were not associated with a significant protein peak when the toxin was subjected to preparative isoelectric focusing (Fig. 7). In the future, immunochemical experiments using techniques such as rocket immunoelectrophoresis and two-dimensional immunoelectrophoresis will be performed to determine the purity of the *Salmonella* toxin.

The ELISA and the CHO floating cell assay were both very sensitive and were reliable for the detection of the antigenic and biological characteristics of *Salmonella* toxin, respectively. The CHO floating cell assay was capable of detecting as little as 0.01 ng of cholera toxin. This assay was based on the capacity of *V. cholerae* and *E. coli* enterotoxins to increase the adhesiveness of proliferating CHO cells, causing them to accumulate on adherent monolayers (14). In this study, we found that *Salmonella* toxin also causes an increase in the adherence of CHO cell monolayers. It was the accumulation of adherent proliferating cells to the monolayers that accounted for the smaller number of CHO floating cells found in the medium. Therefore, CHO cell cultures not treated with cholera toxin or *Salmonella* toxin had a larger number of floating cells than CHO cell cultures treated with either toxin. The CHO floating cell assay proved to be a convenient and reliable tool for measuring the biological activities of both *Salmonella* and cholera toxin.

The absence of biological activity does not always indicate the absence of antigen. With this in mind, we used the ELISA to detect the presence of the *Salmonella* toxin antigens shared with cholera toxin in a quantitative manner. The ELISA was capable of detecting quantities of cholera toxin as small as 0.37 ng. Toxin preparations from both *V. cholerae* and *Salmonella* strains were utilized as antigens in the ELISA, along with culture medium controls. Our results indicated that only the antigen preparations of cholera toxin and *Salmonella* toxin in the presence of specifically purified cholera antitoxin were able to elicit a positive ELISA response. Therefore, the specificity of the ELISA is directly related to the specificity of the specifically purified cholera antitoxin. Nonspecific adsorption of cholera antitoxin to the microtiter plate wells was prevented by phosphate-buffered saline-Tween buffer, in which the cholera antitoxin was diluted. The detection of *Salmonella* toxin by using the ELISA with cholera antitoxin was further evidence for the cross-reactivity that exists between cholera toxin and *Salmonella* toxin.

We attempted to determine the role of MTC in the synthesis and release of *Salmonella* toxin and studied some of the biochemical, biological, and antigenic characteristics of this toxin by using the CHO floating cell assay and the ELISA. The coupling of an antigenic assay with a biological assay was very advantageous in the study of *Salmonella* toxin. Both the CHO floating cell assay and the ELISA have proven to be very sensitive and reliable. The addition of MTC increased the amount of *Salmonella* toxin present in filtrates but had the reverse effect on sonic extracts of *Salmonella* cells. The antigenicity of *Salmonella* toxin was relatively heat stable, although the antigen contained in *Salmonella* filtrates was more heat stable than the antigen contained in cell sonic extracts. Heating destroyed virtually all of the biological activity of *Salmonella* toxin in both filtrates and sonic extracts. The toxin had an isoelectric point in the range from pH 4.3 to pH 4.8 and an estimated molecular weight of at least 110,000. Although we present data which suggest that *Salmonella* toxin may be a protein or at least contain a protein moiety, a further investigation will be conducted before a definitive statement can be made. When *Salmonella* toxin has been purified successfully, we will reexamine this toxin regarding its isoelectric point and molecular weight. With exception of the range for its isoelectric point, its molecular weight, and its low concentration in filtrates and sonic extracts, *Salmonella* toxin appears to be very similar in biological and antigenic characteristics to cholera toxin, which has an isoelectric point near pH 7 and a molecular weight of 84,000 and is produced in relatively large concentrations by some strains. We feel that the antigenic and biological assays described here will provide an effective basis for extending the study of the characteristics, mechanism of toxin release, and role of *Salmonella* toxin in the pathogenesis of salmonellosis.

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LITERATURE CITED

1. **Bennett, V., and P. Cuatrecasas.** 1975. Mechanism of action of *Vibrio cholerae* enterotoxin. J. Membr. Biol. **22**:1–52.
2. **Bio-Rad Laboratories.** 1977. Bio-Rad protein assay. Technical bulletin 1051. Chemical Division, Bio-Rad Laboratories, Richmond, Calif.
3. **Dafni, Z., R. B. Sack, and J. P. Craig.** 1978. Purification of heat-labile enterotoxin from four *Escherichia coli* strains by affinity immunoadsorbent: evidence for similar subunit structure. Infect. Immun. **22**:852–860.
4. **Finkelstein, R. A., M. K. LaRue, D. W. Johnson, M. L. Vasil, G. J. Cho, and J. R. Jones.** 1976. Isolation and properties of heat-labile enterotoxin(s) from enterotoxigenic *Escherichia coli*. J. Infect. Dis. **133**:S120–S137.
5. **Finkelstein, R. A., and J. J. LoSpalluto.** 1970. Production of highly purified choleragen and choleragenoid. J. Infect. Dis. **121**:S63–S72.
6. **Gemski, P., A. D. O'Brien, and J. A. Wohlhieter.** 1978. Cellular release of heat-labile enterotoxin of *Escherichia coli* by bacteriophage induction. Infect. Immun. **19**:1076–1082.
7. **Guerrant, R. L., L. L. Bruton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholera* and *Escherichia coli*. Infect. Immun. **10**:320–327.
8. **Hejtmancik, K. E., J. W. Peterson, D. E. Markel, and A. Kurosky.** 1977. Radioimmunoassay for the antigenic determinants of cholera toxin and its components. Infect. Immun. **17**:621–628.
9. **Isaacson, R. E., and H. W. Moon.** 1975. Induction of heat-labile enterotoxin synthesis in enterotoxigenic *Escherichia coli* by mitomycin C. Infect. Immun. **12**:1271–1275.
10. **Koupal, L. R., and R. H. Deibel.** 1975. Assay, characterization, and localization of an enterotoxin produced by *Salmonella*. Infect. Immun. **11**:14–22.
11. **Kunkel, S. L., and D. C. Robertson.** 1979. Purification and characterization of the heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli*. Infect. Immun. **25**:586–596.
12. **Kurosky, A., D. E. Markel, B. Touchstone, and J. W. Peterson.** 1976. Chemical characterization of the structure of cholera toxin and its natural toxoid. J. Infect. Dis. **133**:S14–S22.
13. **Molina, N. C., and J. W. Peterson.** 1980. Cholera toxin-like toxin release by *Salmonella* species in the presence of mitomycin C. Infect. Immun. **30**:224–230.
14. **Nozawa, R. T., T. Yokota, and S. Kuwahara.** 1978. Assay method for *Vibrio cholerae* and *Escherichia coli* enterotoxins by automated counting of floating Chinese hamster ovary cells in culture medium. J. Clin. Microbiol. **7**:479–485.
15. **Peterson, J. W., J. J. LoSpalluto, and R. A. Finkelstein.** 1972. Localization of cholera toxin in vivo. J. Infect. Dis. **126**:617–628.
16. **Peterson, J. W., and P. D. Sandefur.** 1979. Evidence of a role for permeability factors in the pathogenesis of salmonellosis. Am. J. Clin. Nutr. **32**:197–209.
17. **Sandefur, P. D., and J. W. Peterson.** 1976. Isolation of skin permeability factors from culture filtrates of *Salmonella typhimurium*. Infect. Immun. **14**:671–679.
18. **Sandefur, P. D., and J. W. Peterson.** 1977. Neutralization of *Salmonella* toxin-induced elongation of Chinese hamster ovary cells by cholera antitoxin. Infect. Immun. **15**:988–992.
19. **Voller, A., D. Bidwell, and A. Bartlett.** 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p. 506–512. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology. American Society for Microbiology, Washington, D.C.